

## Transformation efficiencies and expression patterns of a series of truncated *GS<sub>I-2</sub>* promoter/*GUS* transgenes in maize

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One isoform of maize glutamine synthetase, encoded by *GS<sub>I-2</sub>*, is localized exclusively in the maternal tissues of the developing kernel. Previously, we have demonstrated the ability of the proximal 664 base pair 5' upstream portion of *GS<sub>I-2</sub>* to drive maternal tissue-specific GUS expression in transgenic maize kernels (Muhitch et al. Plant Sci. 163: 865–872). In this report, a series of *GS<sub>I-2</sub>* promoter/*GUS* reporter transgenes, progressively truncated from the 5' end of the full length 664 base pair promoter, were evaluated for transformation efficiency and their ability to drive tissue-specific gene expression in transgenic maize. Analysis of transgene integration and expression

suggests that *GS<sub>I-2</sub>*/*GUS* transgenes were incorporated efficiently into the maize genome, but were not expressed efficiently in maize cells. Truncation of the promoter from –664 to –394, –206 or –72, relative to the putative transcription start site, resulted in the loss of tissue specific expression within the kernels of transformed plants. Among the truncated series, moderate staining was exhibited by the –394 promoter/*GUS* gene transformants, stronger staining was found in –206 promoter/*GUS* gene transformants, but relatively weak and variable staining occurred in plants transformed with the –72/*GUS* gene. Likely explanations for these observations are considered.

### Introduction

Ammonium nitrogen that has been assimilated into organic compounds is released and re-assimilated during the processes of photorespiration, seed germination, senescence, and seed fill (Lea and Ireland 1999). Glutamine synthetase (GS) is a key enzyme involved in the capture and re-capture of ammonia, catalysing its incorporation into the  $\gamma$  carboxyl group of glutamate to form glutamine (Cren and Hirel 1999). Maternal tissues that surround developing seeds are often rich in GS (Duffus and Rosie 1978, Murray and Kennedy 1980, Garg et al. 1985, Peoples et al. 1985, Muhitch 1988). In the case of maize, a distinct GS isozyme, designated as GS<sub>p1</sub>, resides within these seed-associated, maternal tissues (Muhitch 1989, Muhitch et al. 1995). Rastogi et al. (1998) presented evidence that, of the six maize GS genes (Sakakibara et al. 1992, Li et al. 1993) *GS<sub>I-2</sub>* encodes the GS<sub>p1</sub> isozyme. Further proof was provided by demonstrating that the promoter from *GS<sub>I-2</sub>* was capable of driving tissue specific gene expression exclusively within the maternal tissues of transgenic maize

kernels (Muhitch et al. 2002). In this current report, the effects of truncation from the 5' end of the *GS<sub>I-2</sub>* promoter on the transformation efficiency and tissue specificity of transgene expression are presented. Likely explanations for the observed low efficiencies of GUS expression in calli of maize lines transformed with the *GS<sub>I-2</sub>*/*GUS* transgenes, as well as for the apparent lower retention of GUS expression in plants regenerated from calli transformed with the full length *GS<sub>I-2</sub>*/*GUS* construct, are considered.

### Materials and methods

Construction of the full length *GS<sub>I-2</sub>*/*GUS* transgene, which includes the 5' UTR and first two introns from *GS<sub>I-2</sub>*, has been described earlier (Muhitch et al. 2002). To produce the three truncated plasmid constructs (pGS394, pGS206, pGS72), a *SalI*/*Bam*HI fragment containing the promoter was excised from the full length construct and a *SalI* site introduced at the indicated positions by PCR. The truncated transgenes were then

Abbreviations – GS, glutamine synthetase; GUS,  $\beta$ -glucuronidase.

re-cloned into the pUC19 vector at the *SalI/BamHI* site and sequenced. With the exception of the 5' deletions in the promoter, therefore all three truncated *GS<sub>1-2</sub>/GUS* genes were identical to pGS664, the original full length construct (see Fig. 1).

Transformation of Hi-II calli was performed at the Plant Transformation Facility, Ames, Iowa, USA, as described by Frame et al. (2000). Screening, plant regeneration, growth conditions, GUS histochemistry and Southern methodology were performed as previously described (Muhitch et al. 2002). PCR screening of resistant calli was performed with two sets of primers, one which spanned the full length *GUS/NOS* gene and the second which spanned from just inside the end of the 5' end of the *GS<sub>1-2</sub>* promoter or promoter fragment to just inside the 5' end of *GUS*. Lines were considered successfully transformed if they exhibited at least moderate, consistent GUS staining in tissue samples from at least 3 regenerated sibling plants derived from a single callus line. Control samples of B73 inbred tissues were incubated in parallel to transgenic samples to check for endogenous GUS activity (Muhitch 1998).

## Results

### *GS<sub>1-2</sub>* promoter truncation series

Bialophos-resistant calli recovered from particle bombardment with the full length *GS<sub>1-2</sub>/GUS* gene or one

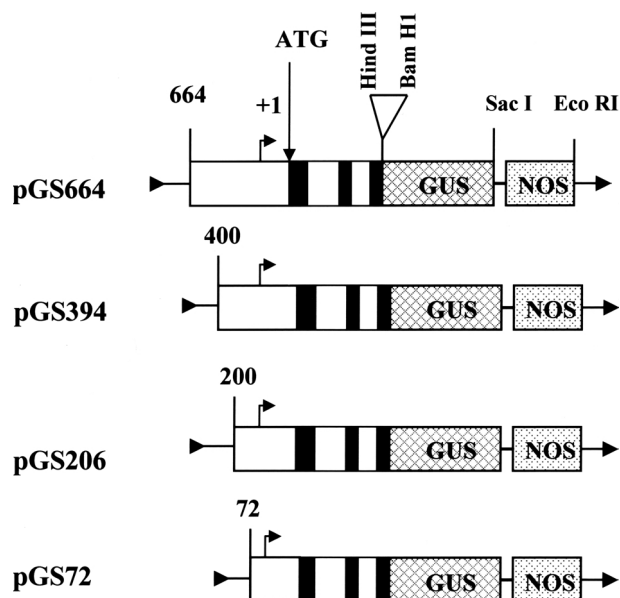


Fig. 1. *GS<sub>1-2</sub>* promoter/reporter gene constructs. The *GS<sub>1-2</sub>* segment from 664 bp (upstream of the putative transcription start site) to the *HindIII* site within the third exon, was fused in frame to  $\beta$ -glucuronidase (*GUS*) to create the full length transgene vector with a *SalI* restriction site flanking the 5' end. The upstream region of the *SalI/BamHI* promoter fragment was truncated to -394, -206 and -72 to generate a 5' deletion series, pGS394, pGS206 and pGS72, respectively. The black rectangles denote exonic regions. NOS, nopaline synthase terminator.

of its truncated versions (Fig. 1) were initially screened using both PCR and GUS histochemical staining (see Materials and methods). After the first round of screening, regeneration and analysis of plants transformed with any of these four constructs, it became apparent that GUS staining in the bialophos-resistant calli was a necessary prerequisite to GUS staining in the regenerated transformants. Therefore, further rounds of bombardment with the truncation series constructs were screened using histochemical GUS assays of bialophos-resistant calli alone.

The relative efficiency of maize transformation with the *GS<sub>1-2</sub>/GUS* truncation series constructs was examined (Table 1). The initial transformation efficiency, i.e. the recovery of bialophos-resistant calli following particle bombardment, varied from 7.6 to 12.8% (Table 1). Furthermore, incorporation of the transgene into the maize genome, regeneration of transformed calli, and seed set efficiency did not vary appreciably among the 4 constructs (Table 1). Expression of GUS activity within transformed calli, as measured by histochemical staining, was relatively low for all four lines when compared to transgene incorporation. Since GUS expression in bialophos-resistant calli was nevertheless a prerequisite of successful *GS<sub>1-2</sub>/GUS* transformation in regenerated maize plants, we also examined the rate of retention of GUS expression when calli were regenerated into plants. In contrast to the other transformation parameters examined, here there appears to be a difference among the plants transformed with different length promoters, in that the calli expressing GUS, after being bombarded with the full length pGS664 gene construct, were less likely to produce plants expressing GUS than were calli bombarded with truncated versions of the gene (Table 1). The overall transformation efficiency, that is, the number of successfully transformed, regenerated, GUS-expressing maize lines per the total number of calli bombarded, ranged from 0.2 to 0.7% (Table 1).

Integration of the transgenes into the maize genome was determined and copy number estimates were made by DNA blot analysis. Transgene copy number estimates ranged from 4 to 26, 26–37, and 51–73 for plants transformed with pGS394, pGS206 and pGS72, respectively. Copy numbers of pGS664-transformed lines have been previously estimated at 11–22 (Muhitch et al. 2002). All of the transformants contained additional, mostly higher molecular weight bands as well, particularly those lines transformed with pGS72 (see Fig. 2).

Seed histochemical GUS staining in transgenic maize plants, transformed with the full length pGS664 construct, was confined to the maternal tissues of the developing maize kernels (Fig. 3A) as found previously (Muhitch et al. 2002). In contrast, truncation of the promoter from 664 to 394 bp upstream of its putative transcription start site resulted in GUS staining throughout all of the kernel tissues (Fig. 3B). Staining in the kernels of plants transformed with the pGS394 construct varied somewhat in strength, but in general was quite strong, resulting in deep blue staining of the pedicel,

Table 1. Efficiency of transformation of maize with *GS<sub>1-2</sub>/GUS* heterologous gene promoter deletion series. Initial transformation efficiency (% of bombarded calli recovered as bialophos resistant), incorporation of the intact gene (# of bialophos-resistant calli containing both promoter and GUS fragments of predicted length upon PCR analysis/total analysed), expression of GUS in calli (# of bialophos-resistant calli expressing GUS activity/total analysed), regeneration of calli (# of bialophos-resistant calli lines, either containing intact transgene or expressing GUS, successfully regenerated to plants/total attempted), seed set efficiency (% of bialophos-resistant regenerated plant lines setting seed/total analysed), regenerated lines retaining GUS (# of regenerated plant lines expressing GUS/GUS expressing bialophos-resistant calli that regenerated), overall transformation efficiency (# of regenerated plant lines expressing GUS/total number of calli bombarded) were determined. Values in parentheses are percentages.

Gene	Initial transformation efficiency	Incorporation of intact gene	Expression of GUS in calli	Regeneration of calli from selected events	Seed set efficiency	Regenerated lines retaining GUS	Overall transformation efficiency
pGS664	7.6%	33/50 (66)	21/135 (16)	46/61 (75)	70%	4/16 (25)	4/1842 (0.2)
pGS394	7.8%	23/41 (56)	20/96 (21)	34/34 (100)	76%	9/12 (75)	9/1244 (0.7)
pGS206	12.8%	35/48 (73)	16/146 (11)	40/57 (70)	82%	7/11 (63)	7/1180 (0.6)
pGS72	8.7%	47/97 (48)	20/198 (10)	55/85 (65)	81%	6/8 (75)	6/2275 (0.3)

pericarp, endosperm and embryo after only 1 h of incubation. As with transgenic maize transformed with the full length construct (Muhitch et al. 2002), GUS staining in pollen was evident (Fig. 3C). In addition, plants transformed with pGS394 also exhibited weak GUS staining in silks and husks (Fig. 3D) as well as moderate staining in roots, leaves, and anther glumes (Fig. 3E,F,G, respectively). Additional truncation of the *GS<sub>1-2</sub>* promoter to approximately 200 bp 5' of the putative transcription start site (pGS206), resulted in GUS staining throughout the kernel tissues that was even stronger than that found for plants transformed with pGS394 (Fig. 3H). In contrast to plants transformed with pGS394, those transformed with pGS206 exhibited

strong silk and husk staining (Fig. 3I), along with stronger staining in leaves, roots, and anther glumes (not shown). Finally, maize plants transformed with the pGS72 construct exhibited highly variable expression patterns. For instance, two lines were recovered that had constitutive GUS staining in all kernel tissues except for the embryo, while a third line exhibited staining predominantly in the scutellar tissue of the embryo (Fig. 3J). Non-transformed control tissue samples were completely free of stain (Fig. 3K,L).

#### *GS<sub>1-2</sub>* instability when expressed in *trans*

As noted, calli that exhibited GUS activity after being transformed with the full length construct appeared less efficient at retaining GUS activity in regenerated plants than calli transformed with truncated versions of the gene (Table 1). To explore this observation further, three maize lines, transformed with pGS664, that exhibited GUS in calli, but not in the subsequently regenerated plants (GUS-) were compared with three other lines that retained GUS activity in both the calli and in the subsequently regenerated plants (GUS+). Blots of *Eco*RI-digested DNA samples probed with the labelled *GUS/NOS* gene revealed that both expressing and nonexpressing plant samples contained an appropriate-sized band corresponding to the intact, un-rearranged *GUS/NOS* fragment (Fig. 4A). The GUS- lines, however, tended to have more intense and greater numbers of additional higher molecular weight bands than did the GUS+ lines (Fig. 4A). When the DNA samples were digested with the methylation-sensitive restriction endonuclease *Hpa*II (Fig. 4B), lanes containing samples from GUS- plants exhibited more large-sized bands than the lanes containing samples from GUS+ plants, indicative of increased methylation. Ideally, in the absence of methylation, no *GUS/NOS* cross-reacting bands should have been retained on the gel after separating the *Hpa*II DNA fragments.

#### Discussion

The *GS<sub>1-2</sub>* gene encodes an isozyme of glutamine synthetase that is strongly expressed in the pedicel of developing maize seeds (Rastogi et al. 1998) and whose promoter is

1 2 3 4 5 6 7 8 9 10 11

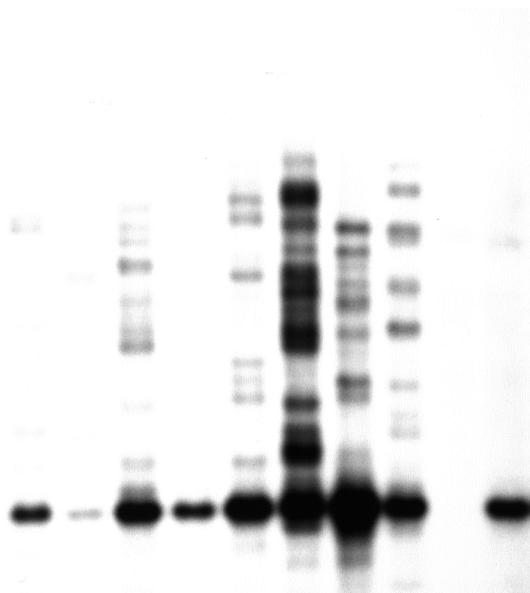


Fig. 2. DNA blot analysis of leaves from maize lines transformed with one of the three truncated promoter constructs, pGS394 (lanes 1-3), pGS206 (lanes 4-6) or pGS72 (lanes 7-9). Lane 10, B73 non-transformed (negative control). Lane 11, *GUS/NOS* fragment (positive control). Each DNA sample (5 µg) was from an independent transformation event and was digested with *Eco*RI. Blots were probed with <sup>32</sup>P-labelled *GUS/NOS* fragment.



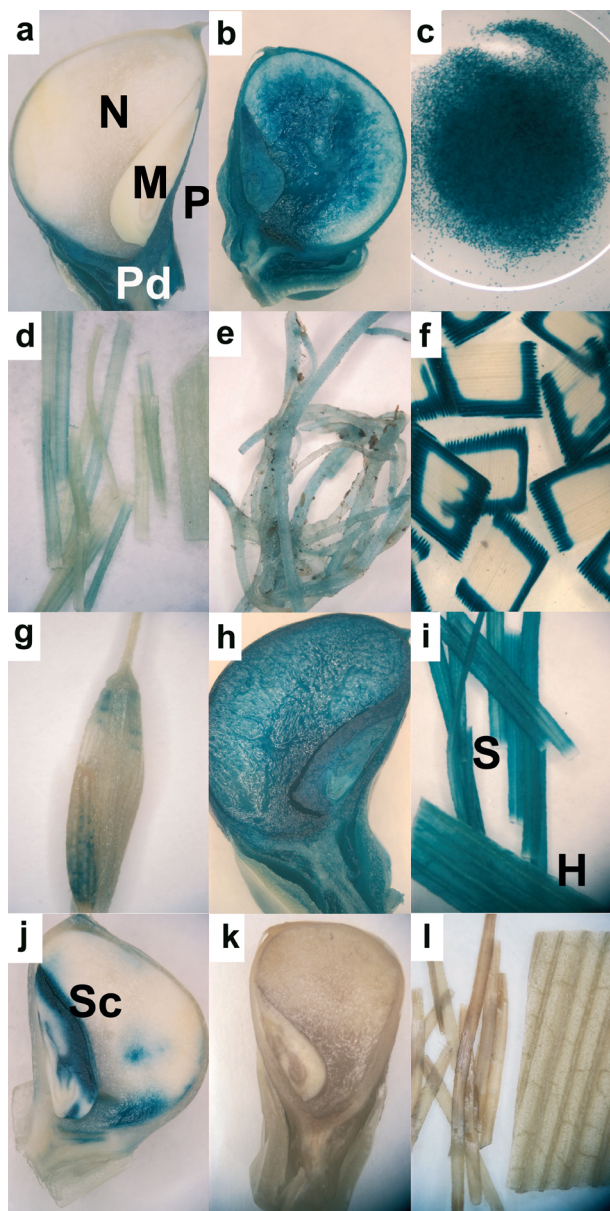


Fig. 3. Histochemical GUS staining of transgenic maize tissues. Panel (a), kernel from plant transformed with the full length *GS<sub>1-2</sub>* promoter/*GUS* construct (pGS664), incubated for 6 h. Panels (b)–(g), samples from plants transformed with the pGS394 construct; (b), kernel, incubated 1 h; (c) pollen, incubated 4 h; (d) silks and husks, incubated 16 h; (e) roots, incubated 16 h; (f) leaf, incubated 16 h (leaf segments were cleared of chlorophyll with ethanol prior to being photographed); (g) anther, incubated 16 h (staining distal to the base is from underlying pollen). Panels (h)–(i) samples from plants transformed with the pGS206 construct; (h) kernel, incubated 1 h; (i) silks and husks, incubated 1.5 h. Panel (j) kernel from plant transformed with pGS72, incubated 16 h. Panels (k) and (l), samples from control, non-transformed B73 plants; (k) non-transformed B73 kernel, incubated 16 h; (l) silks and husks, incubated 16 h. Pd, pedicel; P, pericarp; N, endosperm; M, embryo; S, silks; H, husk; Sc, scutellum.

capable of driving gene expression exclusively in the maternal tissues of developing seeds of transgenic maize plants (Muhitch et al. 2002). Here, the effects of truncation from

the 5' end of the upstream region of the promoter were examined on transgene expression. The initial transformation efficiency, the rates of incorporation of the *GS<sub>1-2</sub>/GUS* into the maize genome, the regeneration of transformed calli, and the seed set efficiency for all members of the truncation series (including the full length transgene) were all similar to what has been obtained with other transgenes introduced into maize (Frame et al. 2000) and are typical of what is routinely observed for particle bombardment at the Iowa State Transformation Facility (Kan Wang, personal communication). These data indicate that neither the full length *GS<sub>1-2</sub>/GUS* nor the truncated constructs have deleterious effects on the transformation process itself. However, while the rates of genome incorporation of intact *GS<sub>1-2</sub>/GUS* transgene copies into bialaphos-resistant calli were relatively high (52–77%), calli expression of GUS activity, as measured by histochemical staining, was much lower (9–14%). This is in sharp contrast, for example, to 78% of bialaphos-resistant maize calli expressing the *GUS* transgene when driven by CaMV 35S promoter (Frame et al. 2002). The difference suggests that while the *GS<sub>1-2</sub>/GUS* gene is incorporated efficiently into the maize genome, it is not expressed efficiently, in the herbicide-resistant calli.

In contrast to the full length *GS<sub>1-2</sub>* promoter, which directed tissue-specific GUS expression within developing kernels (Fig. 3A), all three of the truncated versions of the gene exhibited more general expression patterns. That the removal of the 5' end of the *GS<sub>1-2</sub>* promoter from –664 to –394 alone resulted in the loss of tissue specificity suggests that there may be critical *cis* element(s) within this region that regulate maternal seed tissue-specific gene expression. The only *cis* elements known to date in maize, that are involved in maternal seed tissue gene expression, are the *myb* protein binding sites that regulate flavanoid biosynthesis (Paz-Ares et al. 1987, Grotewold et al. 1994). Analysis of the 5' upstream region of the *GS<sub>1-2</sub>* gene using a plant transcription factor homology database (Higo et al. 1999) revealed that it contains a number of potential *myb* factor binding sites. Of these, only one, *myb1* (Baranowskij et al. 1994), was found exclusively in the –664 to –398 bp region, occurring at –664 and also in the minus strand at –470 (not shown). Whether *myb1* or some other, as of yet unidentified, *trans* factor recognition motif in this region directs tissue specific gene expression remains to be determined.

Within the truncations series, GUS expression was stronger in the plants transformed with pGS206 than in those transformed with pGS394. It is suggested that the elimination of the segment –394 to –206 may remove additional negative elements that normally suppress gene expression in non-maternal seed tissues. In contrast, when the promoter was truncated to –72, the resulting transformed plants showed relatively weak and highly variable GUS expression patterns. The reason for the increased variability is not known, although the elimination of a CAAT box (–165 to –162) or other critical elements may render the regulation of pGS72 more

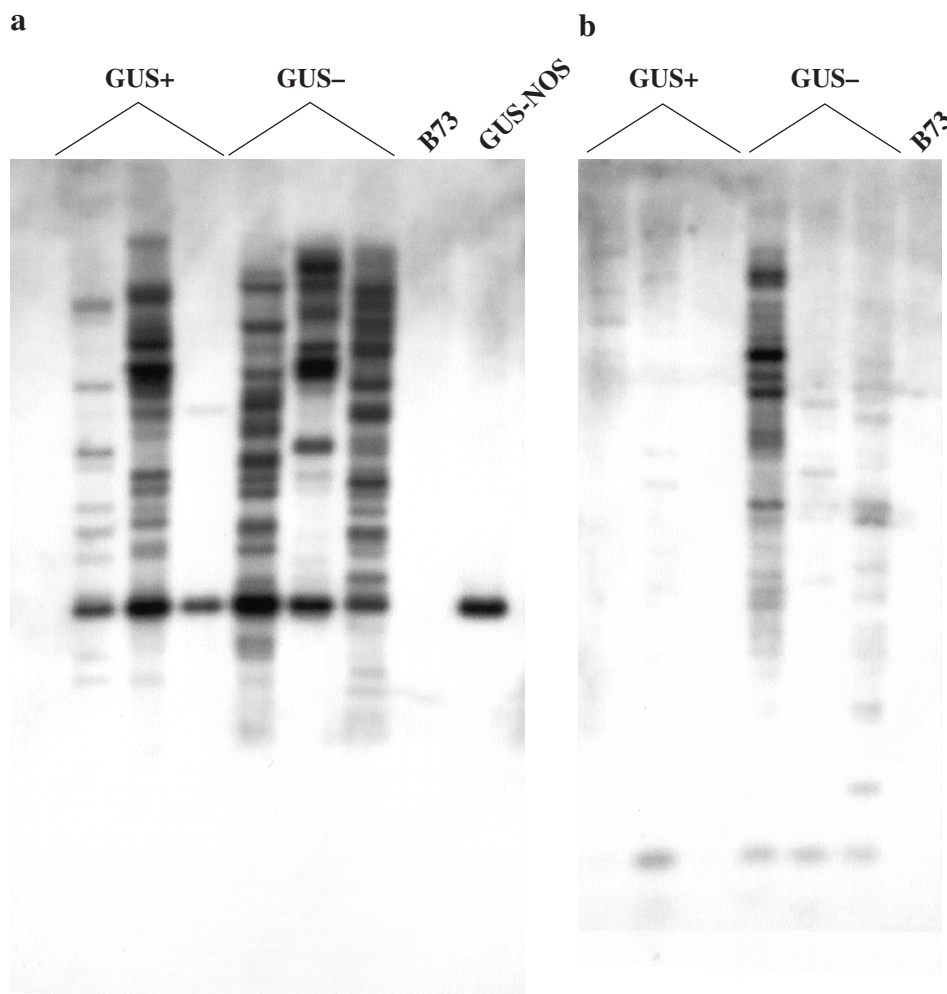


Fig. 4. DNA blot analysis of leaves from maize lines retaining GUS activity (GUS+) or not retaining GUS activity (GUS-) after regeneration from GUS-expressing, bialaphos-resistant callus. Duplicate samples (5 µg) were digested with either *Eco*RI (panel a) or *Hpa*II (panel b). Controls and probe were as described in Fig. 2.

susceptible to positional or other epigenetic effects (Down et al. 2001).

As previously stated, the percentage of calli expressing GUS when transformed with the *GS<sub>1-2</sub>/GUS* gene was relatively low. Moreover, calli expressing GUS after being transformed with the full length construct appeared to more frequently fail to retain GUS expression in regenerated plants than did those calli transformed with a truncated version of the transgene (Table 1). These observations suggest that, *in trans*, the *GS<sub>1-2</sub>/GUS* genes, particularly those containing the full-length promoter, are subject to gene instability or silencing. Southern blot analysis of the *GS<sub>1-2</sub>/GUS* transformants revealed complex patterns of transgene integration (Fig. 2 and Muhitch et al. 2002). It has been well established that particle bombardment often results in the insertion of multiple transgenes copies into a single locus, with many of the copies undergoing rearrangements and additional modifications that can cause methylation or other epigenetic effects resulting in gene silencing

(Matzke et al. 1994, Lakshminarayan et al. 2000, Mehlo et al. 2000). Comparative DNA blot analysis (Fig. 4) of pGS664-transformed maize lines exhibiting GUS activity in calli, but not in the plants regenerated from those calli (GUS-) and of lines that continued to exhibit GUS activity following regeneration (GUS+) suggests that in regenerated GUS- lines, the transgenes are stable, but are undergoing increased methylation. Whether this methylation is a cause or effect of gene silencing is unknown. Admittedly, any interpretation of differential methylation patterns is complicated by the presence of multiple, rearranged copies of the transgenes. It is possible, for example, that the truncated promoters are simply more readily influenced by either the flanking genomic DNA or by the juxtaposed transgene fragments than is the full-length promoter. If so, this could explain both the truncated promoters' lack of tissue specific expression as well as their predisposition for maintaining expression in regenerated plants. The observed variability of expression patterns in different lines transformed with

the most truncated construct (pGS72) is consistent with this suggestion. We are currently incorporating the full length *GS<sub>1-2</sub>/GUS* gene construct into maize using *Agrobacterium tumefaciens*-mediated transformation (Frame et al. 2002), a method that results in only a single or a few transgene copies being incorporated into the genome with little modification (Ishida et al. 1996), in an effort to gain additional understanding of the regulation of the *GS<sub>1-2</sub>* promoter expression in a transgenic context.

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